

AWARD NUMBER: W81XWH-14-1-0256 (MD130059)

TITLE: Development of Orally Bioavailable Therapeutics by the Chloroplast Expression System to Counter Muscle Degeneration, Weakness, and Fibrosis in DMD

PRINCIPAL INVESTIGATOR: Elisabeth Barton

CONTRACTING ORGANIZATION: University of Florida
GAINESVILLE FL 32611-0001

REPORT DATE: November 2016

TYPE OF REPORT: Annual Technical

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE November 2016		2. REPORT TYPE Annual		3. DATES COVERED 1Aug2015 - 31Oct2016	
4. TITLE AND SUBTITLE Development of Orally Bioavailable Therapeutics by the Chloroplast Expression System to Counter Muscle Degeneration, Weakness, and Fibrosis in DMD				5a. CONTRACT NUMBER W81XWH-14-1-0256	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Elisabeth Barton, PhD E-Mail: erbarton@ufl.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) UNIVERSITY OF FLORIDA 207 GRINTER HALL GAINESVILLE FL 32611-0001				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Patients with DMD suffer from progressive muscle weakness and damage, resulting in fibrotic replacement. The goal of this project is to evaluate the therapeutic potential of the anti-fibrotic agents, ACE2/Ang(1-7), when produced in plants using a chloroplast expression system. Lyophilized plant material was delivered by oral gavage to the mdx mouse model for DMD. Initial studies were done to ensure that the plant material and protein was orally bioavailable. Further, additional studies confirmed that ACE2 protein accumulated in the circulation over the course of the treatment. Functional assessment of mice treated for 2 weeks showed improved strength in the diaphragm muscles. However, by 2 months of treatment the benefits were reduced back to untreated controls. We are continuing to analyze more outcome measures for these animals in order to understand if the limited benefit is due to delivery issues, or if the protein itself is only transiently beneficial. Nevertheless, we believe that this delivery strategy may provide a new way to introduce therapeutic proteins for treating neuromuscular disease.					
15. SUBJECT TERMS DMD, chloroplast expression system, renin-angiotensin pathway, fibrosis, skeletal muscle function.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 10	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	9
5. Changes/Problems.....	9
6. Products.....	9
7. Participants & Other Collaborating Organizations.....	10
8. Special Reporting Requirements.....	10
9. Appendices.....	10

1. Introduction

The overarching goal of this proposal is to evaluate a novel delivery system as a strategy to treat DMD, using a promising anti-fibrotic therapy, Angiotensin Converting Enzyme 2 and its enzymatic product Angiotensin-(1-7) (ACE2/Ang-(1-7)). Specifically, we have tested chloroplast derived ACE2 and Ang-(1-7) in lettuce to evaluate each agent independently and in combination in the *mdx* mouse model for DMD. Our outcome measures include a standard battery of physiological and morphological assessments, including muscle function (force generation capacity, stiffness, and fragility), extent of fibrosis (Sirius red staining and hydroxyproline content), and muscle stabilization (central nucleation, embryonic myosin expression, serum creatine kinase levels). This comprehensive analysis will provide key pre-clinical data for both the use of Ace2/Ang(1-7) as a treatment for DMD, as well as the potential of this delivery strategy for additional proteins for muscle disease.

2. Keywords

Renin-angiotensin pathway
Angiotensin Converting Enzyme 2
Angiotensin-(1-7)
Fibrosis
Duchenne Muscular Dystrophy
Mdx
Skeletal muscle
Chloroplast expression
Oral bioavailability

3. Accomplishments

The major goals and accomplishments are listed below, organized by the Tasks in the Statement of Work.

Task 1. Tobacco ACE2/Ang(1-7) activity validation (Daniell) (months 1-16):

- 1a. Plant transgenic tobacco seeds and grow (months 1-4, typical growing period; repeated 2 times).
- 1b. Harvest tobacco plants (month 4, 8, 12).
- 1c. Prepare leaf material for protein activity (months 4, 8, 12).
- 1d. Perform immunoblotting to protein quantification (months 1, 4, 8, 12).
- 1e. Perform ACE2 activity assay (months 1, 4, 8, 12).

Plant production and quantification of ACE2 and Ang(1-7) were completed by the Daniell lab. These materials were shipped to the Barton lab for evaluation in the UF mouse colony. Note that the production level of ACE2 by the plants is lower than the other proteins. In this plant line, there was no codon optimization, which impairs the efficiency of translation of mammalian proteins by plants. Future transplastomic plants are under production in order to attempt to boost ACE2 levels.

leaf sample	concentration(μ g/mg)	dry weight (g)
CTB-ACE2	0.2	5.24
CTB-Ang(1-7)	8.64	4.97
CTB-GFP	5.6	1.13

Figure 1. Quantification of therapeutic protein per mg plant material by immunoblotting.

Task 2. ACE2/Ang(1-7) Pharmacokinetics (Daniell/Barton) (months 1-2):

- 2a. Oral dosing of C57 mice for muscle biodistribution (N=64) (months 1-2).
- 2b. Protein quantification in muscles and blood (months 1-2).

Confirmation of Circulating ACE2 after feeding was performed using a fluorescent reporter for ACE2 enzymatic activity. Shown in Figure 2 is the comparison of rates between N=3 fed mice and 1 naïve control 5 hours post gavage. Fed ACE2 activity levels were more than 2-fold higher than endogenous levels with 1 oral gavage of 25 mg plant material. We next measured the changes in circulating ACE2 activity over the course of 2 months treatment in mature Cdx mice. Blood was obtained at trough (24 hours after last dosing and immediately before subsequent dose). As shown in Figure 3, there is an accumulation of ACE2 in the circulation out to 6 weeks (the longest measured thus far). This provides confidence that the oral gavage procedures work well, and that oral bioavailability of the plant derived proteins occurs.

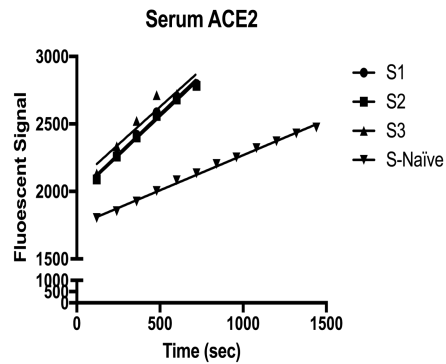


Figure 2. Fluorescent based ACE2 activity assay. Average slope of test lines was 1.15 FI/sec vs Naïve sample at 0.521 FI/sec.

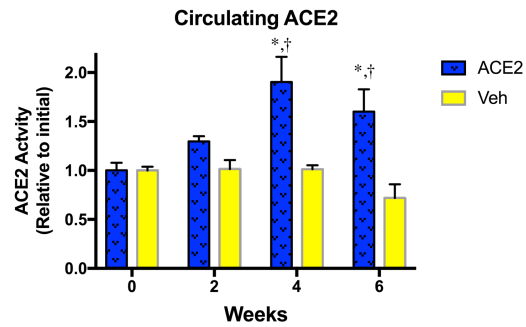


Figure 3. Cumulative ACE2 activity relative to initial for N=6 treated and N=4 control muscle. *, $P < 0.05$ vs initial for the same animal. †, $P < 0.05$ vs Vehicle (Veh) for the same timepoint.

Biodistribution studies were performed using GFP reporter plants by the Daniell lab and recently published (Xiao et al, 2016). While this was not the direct task, and was supported by related funds in the Daniell lab, the study demonstrated nicely that plant-based proteins could enter the circulation and also enter skeletal muscle (Figure 4). This was an important step forward in the project. Also note that the current study uses plants with the CTB extension. Interestingly PTD-GFP appears to have better exposure in muscle. The Daniell lab is working on re-designing their protein constructs to incorporate this new extension. While outside of the scope of this project, future studies are likely to incorporate the newly designed plant expression systems.

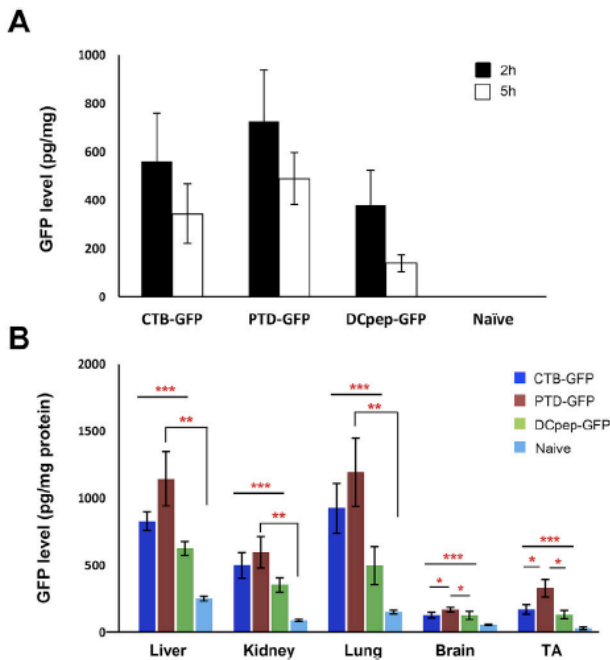


Figure 4. Efficiency of oral delivery and biodistribution of GFP fused with different tags. Serum (A) and tissue (B) GFP levels in mice (N ¼ 6 per group) fed leaf materials expressing CTB-GFP, PTD-GFP and DCpep-GFP. Adult mice were orally fed with leaf materials from transgenic tobacco plants, with the amount adjusted to GFP expression levels, for three consecutive days. A control group (N ¼ 6) kept unfed. Blood samples were collected at 2 and 5 h after last gavage at which, mice were sacrificed and tissue samples were collected for protein isolation. GFP concentration in serum and tissues were measured with ELISA. The data was shown as average \pm SEM. Statistic significance was determined by a paired Student's *t* test, and *p* value less than 0.05 were considered significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.05$ or $P < 0.01$ (CTD, PTD and DCpep versus Naïve). (From Xiao et al, Biomaterials 80 (2016) 68e79)

Task 3. Breeding Cmdx and C57 mice for colony maintenance and expansion (Barton) (months 1-30)

- 3a. Purchase mice (month 1).
- 3b. Breed mice for older age group (month 2-4).
- 3c. Breed mice for younger age group (month 2-3; 5-6).

Mice were purchased from Jackson Labs to establish a breeding colony of Cmdx and strain matched controls. Mice were bred at UF and at Penn (the PIs former institution) to generate 6 month old mice for treatment groups. Mice were bred at UF generate 1 month old mice for treatment groups. Only male mice were used for the treatment groups. To date a total of 276 Cmdx mice and 184 C57Bl10 mice (M and F for both strains) were generated at UF. Active breeding has been tapered to only maintenance in case the need arises to generate more mice for specific conditions.

Task 4. Oral gavage of ACE2/Ang(1-7) in dystrophic mice (Barton) (months 3-14)

- 4a. Dose Ang(1-7) into young mice (N=36) (months 3-9)
- 4b. Dose ACE2 into young mice (N=36) (months 4-10)
- 4c. Dose ACE2/Ang(1-7) into young mice (N=36) (months 5-11)
- 4d. Dose Ang(1-7) into mature mice (N=36) (months 6-12)
- 4e. Dose ACE2 into mature mice (N=36) (months 7-13)
- 4f. Dose ACE2/Ang(1-7) into mature mice (N=36) (months 8-14)

Oral gavage studies have been performed on 102 mice to date, and an additional 24 mice have been used as untreated controls. A total of 8 mice died or were required to be euthanized during the treatment periods. This was due to significant weightloss associated with the phenotype which is incorporated into our humane endpoints criteria.

Task 5. Evaluation of ACE2/Ang(1-7) in dystrophic mice (Barton/Daniell) (months 4-15)

- 5a. Test Ang(1-7) treated and control young mice (months 4-10)
- 5b. Test ACE2 treated and control young mice (months 5-11)
- 5c. Test ACE2/Ang(1-7) treated and control young mice (months 6-12)
- 5d. Test Ang(1-7) treated and control mature mice (months 7-13)
- 5e. Test ACE2 treated and control mature mice (months 8-14)
- 5f. Test ACE2/Ang(1-7) treated and control mature mice (months 9-15)

The primary outcome measure for these studies is muscle strength in the EDL and diaphragms, which will be reported here. Additional measures include morphological analysis of fiber size and fibrosis, circulating creatine kinase, passive force, and cardiac MRI. These additional measures are underway, and will be completed during the upcoming no cost extension period.

Our first study used a very short treatment period of 2 weeks in 6 month old Cmdx animals (Figure 5). Mice were fed daily with ACE2 treatment regimen to determine if any acute functional benefit was achieved. While no significant improvement was observed in EDL muscles, the diaphragms exhibited a significant increase in specific force. A continuation of this study design included a 2 month treatment arm for comparison. However, unlike the benefit observed after short term exposure, there was no significant difference between treated and control muscles (Figure 6)

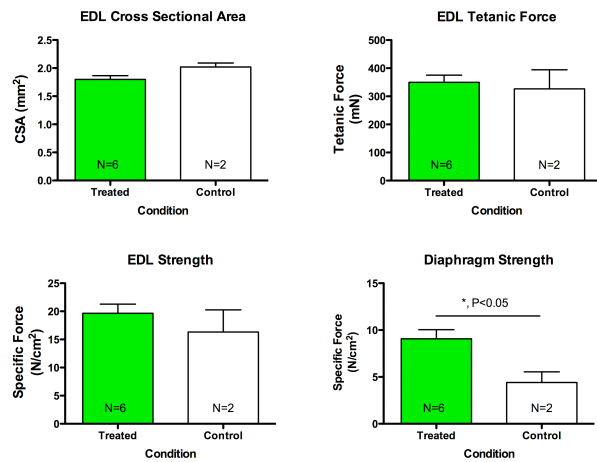


Figure 5. 2 week Treatment of 6 month old male *Cmdx* mice with ACE2 plant. Compared to age matched vehicle treated *Cmdx* controls, there was a significant increase in diaphragm specific force. The EDL muscles did not exhibit any treatment dependent effects.

We went on to test young animals, starting at 4 weeks of age, using Ang(1-7) alone, or a combined treatment. Following 2 months of treatment with Ang(1-7), there was no change in force production in either the diaphragm or the EDL muscles, and strength remained significantly lower than WT (C57) muscles. In order to determine if this was a limitation of the oral bioavailability or an issue with the effects of Ang(1-7), we injected cohorts of WT and *Cmdx* mice with a recombinant adeno-associated virus (AAV) expressing Ang(1-7) regulated by a liver specific promoter. We have used this strategy to produce myostatin related proteins from the liver effectively (Morine et al, 2010 PLoS One. 5(2):e9176). Regardless of the delivery strategy, no benefit of increased Ang(1-7) was observed in the EDL or diaphragms from the *Cmdx* mice (Figure 7). We still need to confirm that there is robust Ang(1-7) production in all of the treated mice, but the result suggests that the acute therapeutic benefit of driving this pathway may be lost in longer term treatment regimens.

To determine if ACE2 and Ang(1-7) combined could provide functional benefit, we treated 1 month old *Cmdx* mice for 1 or 2 months with both plants. For the 1 months group, we delivered plant material 5 times per week. For the 2 month group, we delivered plant material 3 times per week. The rationale for the lower frequency was to minimize the stress of daily oral gavaging, especially with the young animals. As shown in Figure 8, the results were similar to that of Ang(1-7) alone for this duration of treatment. In sum, there was no perceivable benefit of combined ACE2/Ang(1-7) treatment with regard to strength in EDL or diaphragm muscles.

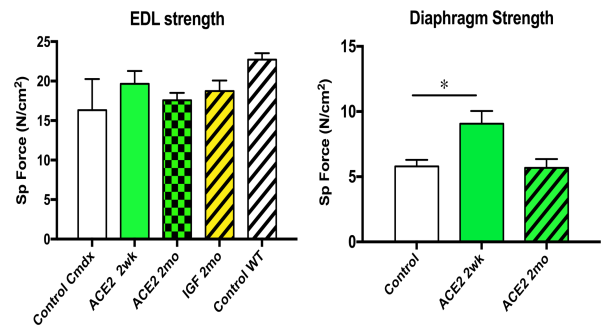


Figure 6. 2 month Treatment of 6 month old male *Cmdx* mice with ACE2 plant. No functional benefit was observed following treatment. 2 wk treatment results are shown for comparison.

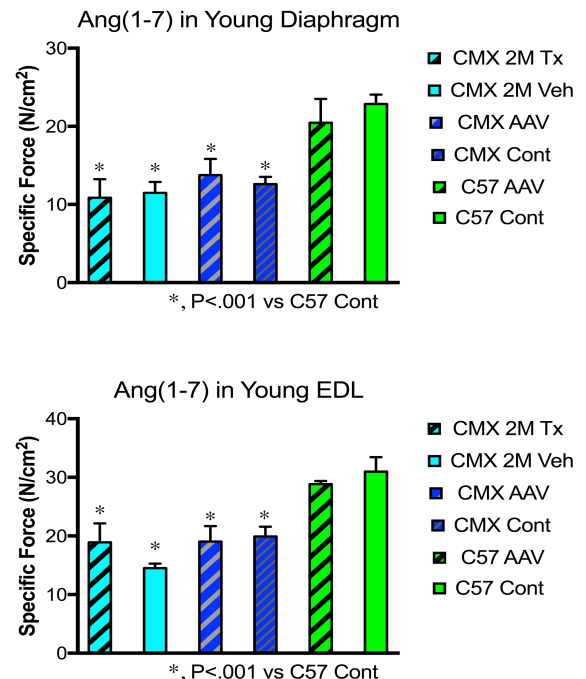


Figure 7. 2 month Treatment of 1 month old male *Cmdx* mice with Ang(1-7) delivered orally by plants or through injection of AAV. No functional benefit was observed following treatment. Statistical comparisons were performed by 1 way ANOVA followed by Tukey post-hoc testing.

Finally, we returned to 6 month old mice, testing the efficacy of combined treatment for 2 months, using feeding 5 times per week. The rationale was to see if the delivery of both the enzyme and the product would help regain the initial observed short-term benefit at this age. As shown in Figure 9, the results were similar to all other longer term trials: there was no improvement in force production following 2 months of treatment in 6 months old mice.

At this point, we are planning to test another cohort of animals with only 2 week treatment to verify if our initial observations were correct. In addition, we are planning to determine if the Mas receptors, which respond to Ang(1-7) are sufficiently abundant in skeletal muscle to afford the benefits originally documented by the Brandan lab (Acuna et al Hum Mol Genet. 2014 Mar 1;23(5):1237-49.). Finally, the complementary outcome measures to assess changes in fibrosis, muscle fiber stability, and fiber size are underway, and will be the focus of the lab in the coming year.

As there were no proposed goals for training and professional development, we have nothing to report.

Task 6. Manuscripts and proposals (Barton/Daniell) (months 12-15)

6a. Prepare manuscript on ACE2/Ang(1-7) delivery (months 12-15).

6b. Prepare proposal for continuation of original project (12-15).

Initial findings were presented by the PI at New Directions in Muscle Biology and Disease meeting in Orlando, FL June 2016 as an oral presentation, and at the European Muscle Conference in Montpellier, France in September 2016.

As the work is still ongoing due to moves and re-establishing the project, a new proposal submission has not been completed.

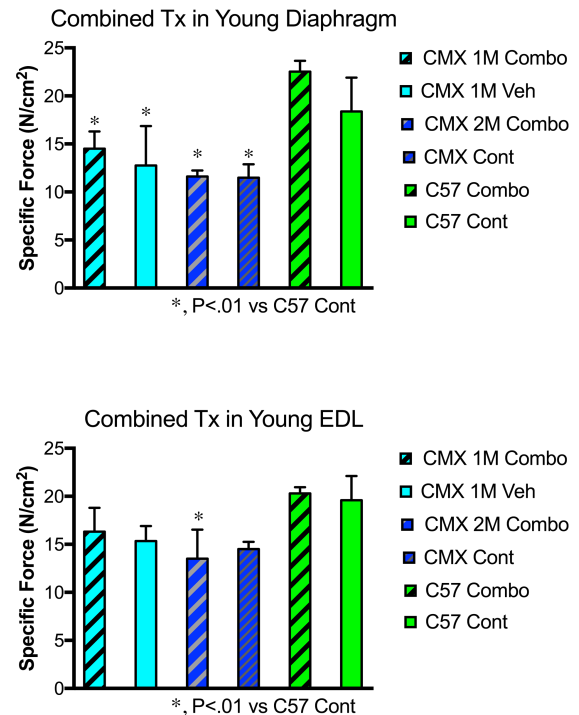


Figure 8. 1 and 2 month Treatment of 1 month old male Cmx mice with ACE2/ Ang(1-7) delivered orally by plants. No functional benefit was observed following treatment. Statistical comparisons were performed by 1 way ANOVA followed by Tukey post-hoc testing.

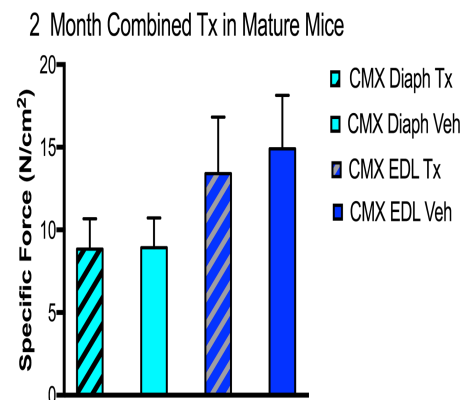


Figure 9. 2 month Treatment of 6 month old male Cmx mice with ACE2/ Ang(1-7) delivered orally by plants. No functional benefit was observed following treatment. Statistical comparisons were performed by 2-tailed T tests.

4. Impact

Development of the principal discipline(s) of the project.

We have optimized delivery of plant derived proteins to mice to ensure that there is exposure to the therapeutic proteins under evaluation.

We have recognized that chloroplast expression of human proteins requires codon optimization in order to produce the proteins efficiently. The Daniell lab is working on new algorithms for codon optimization, and this effort will be used to re-engineer the ACE2 plant lines in increase protein yields.

Impact on other disciplines.

Nothing to report.

Impact on technology transfer.

All of the reagents are under patent protection through either the Daniell or Barton labs. The Daniell lab has entered negotiations with Novo Nordisk on other chloroplast expression projects.

Impact on society beyond science and technology?

Nothing to report.

5. Changes/Problems

Two problems emerged during the funding support. First, there were significant delays in transferring funds from University of Pennsylvania to University of Florida, which caused an approximately 1 year hiatus in progressing to achieve the aims of the proposal. The second problem arises from the possibility that the therapeutic proteins under investigation may not be effective in preventing fibrosis in this animal model. This is, in part, the goal of the grant, and so while the initial results presented here may be negative, there is more to be done to understand why this is the case. Both technical and biological limitations are being probed as underlying causes of these findings.

6. Products

Conference presentations:

E.R. Barton. "Development of Orally Bioavailable Therapeutics by Chloroplast Expression Counters Muscle Weakness in DMD." Oral presentation at New Directions in Biology and Disease of Skeletal Muscle Conference Orlando, FL June 29- July 2, 2016. International meeting.

E.R. Barton. "Development of Orally Bioavailable Therapeutics by Chloroplast Expression Counters Muscle Weakness in DMD" Poster presentation at European Muscle Conference, Montpellier, France, September 2-6, 2016. International meeting.

7. Participants & Other Collaborating Organizations

Name	Role	Person Month	Role in Project	Funding Support
Elisabeth Barton	PI	2	Oversee scientific progress, study design, and data analysis and interpretation	DOD, and others
Henry Daniell	Co-Investigator	1	Oversee plant production and quantification	DOD, and others
Hanqin Lei	Research technician	4	enzymatic assays and molecular biological measurements of serum and tissue	DOD, and other (Barton lab)
Yuhong Xiao	Research associate	4	plant production and quantification	Other (Daniell lab)
Kwang-Chul Kwon	Research associate	2	plant production and quantification	Other (Daniell lab)
Ryan Meyer	Undergraduate student	1	Animal feeding and handling	None, research intern
Ray Spradlin	graduate student	1	Animal feeding and handling	Graduate stipend, UF
Michael Matheny	Research technician	2	Muscle functional measurements	NIH, part of muscle core directed by Barton
Jason Puglise	Research technician	1	Muscle functional measurements	NIH, part of muscle core directed by Barton

8. Special Reporting Requirements

NA

9. Appendices

NA